Anatomical and biochemical changes during root formation in oak and apple shoots cultured *in vitro* *

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Summary — The events leading to adventitious root formation in *in vitro* shoot cultures of *Quercus robur* L clone NL3 and *Malus* M9 Jork are described. Significant cellular changes were observed as early as 1–2 d after auxin treatment: in both species, some cambial and vascular parenchyma cells exhibited prominent nuclei and dense cytoplasm, and the first cell divisions were occasionally observed. Meristematic activity had increased by d 4, and meristemoids became individualized 1 or 2 d later. Roots emerged from the shoots by d 8 in oak and d 10 in apple. The vascular systems of the primordium and shoot joined just before root emergence. In oak shoots, peroxidase activity was similar in auxin-treated and untreated shoots during the first 4 d after treatment, but subsequently increased in treated shoots and decreased in untreated shoots. In auxin-treated shoots, peroxidase activity stabilized after d 6.

anatomy / biochemistry / rooting / oak / apple tree = *Malus* sp

Résumé — Changements anatomiques et biochimiques se produisant durant l'enracinement de tiges de chêne et de pommier cultivées *in vitro*. Les événements conduisant à la formation de racines adventives sur des cultures *in vitro* de *Quercus robur*, clone NL3, et *Malus* M9 Jork sont décrits. Des modifications cellulaires significatives ont été observées dès 1 ou 2 jours après le traitement auxinique : dans les 2 espèces, des cellules du cambium et du parenchyme vasculaire montrent des noyaux importants et un cytoplasme dense et quelques premières divisions cellulaires sont observées. Au jour 4, l'activité méristématique augmente et des méristémoïdes s'individualisent 1 ou 2 jours après. Les racines émergent des tiges de chêne au jour 8, et pour le pommier au jour 10. Les systèmes vasculaires du primordium et de la tige se rejoignent juste avant l'émergence de la racine. Chez les tiges de chêne, l'activité peroxydase est similaire dans les tiges traitées à l'auxine et non traitées pendant les 4 premiers jours après le traitement, puis elle augmente dans les tiges traitées, et décroît dans les tiges non traitées. Dans les tiges traitées, l'activité peroxydase se stabilise après le jour 6.

anatomie / biochimie / enracinement / chêne / pommier = *Malus* sp

INTRODUCTION

Plant regeneration *in vitro* provides an alternative to rooting cuttings for the propagation of woody plants. However, rooting of *in vitro* produced shoots is often the limiting step during propagation. Two patterns of adventitious root formation on cuttings have been recognized in both herbaceous and woody plants (Hartmann *et al*, 1990). One consists of the direct development of adventitious root primordia from cells associated with or in close proximity to the vascular system. The other is an indirect process in

which adventitious root formation is preceded by the proliferation of undifferentiated cells, which usually starts in the parenchyma of epidermal cells; certain cells within the undifferentiated tissue then become organized and initiate an adventitious root primordium. In general, the direct pattern is found in herbaceous species and easy-to-root woody species (including those examined in this study), and the indirect pattern in difficult-to-root species.

A diversity of factors control morphogenesis *in vitro*, and no theory clearly explains all the responses observed (Halperin, 1986). As a result, current methods of *in vitro* rooting, like current

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methods of *extra vitrum* rooting, are largely empirical. The more highly controlled conditions of microculture should nevertheless facilitate investigation of the biochemical, genetic and physiological bases of rooting.

One approach to decipher the mechanisms of rooting consists of the use of cytological and histological methods to estimate the physiological status of cells involved in the process of primordium formation (Molnar and Lacroix, 1972a, b). This approach has already been applied in studies on the development of adventitious roots by apple microcuttings (Hicks, 1987; Zhou *et al*, 1992), but we have found no references to *in vitro* root formation by oak shoots. In this paper, we compare the histologically observed events leading to *in vitro* root formation in *Quercus robur* clone NL3 and *Malus* "M9 Jork", and report the evolution of peroxidase activity during the *in vitro* rooting of oak shoots.

MATERIALS AND METHODS

Plant material and culture conditions

Oak

Quercus robur clone NL3 shoots were taken from stock multiplication cultures, which were subcultured every 4-wk in Gresshoff and Doy's medium (1972) supplemented with 30 g l⁻¹ sucrose, 6 g l⁻¹ Vitroagar and 0.2 mg l⁻¹ 6-benzylaminopurine (BA). Four-wk-old, 1.5–2.0-cm long shoots from the stock cultures were dipped in 1 g l⁻¹ indolebutyric acid (IBA) solution for 1 min, and then transferred to Murashige and Skoog's medium (1962) (MS) with half-strength macronutrients and no auxin.

Apple

Malus "M9 Jork" shoots were taken from stock multiplication cultures, which were subcultured every 6 wk in a medium consisting of MS salts, 30 g l⁻¹ sorbitol, 7 g l⁻¹ Sigma agar, 1 mg l⁻¹ BA and 0.1 mg l⁻¹ IBA. Six-wk-old, 1–2-cm long shoots from the stock cultures were rooted in Lepoivre's salt medium (Quoirin and Lepoivre, 1977) with half-strength macronutrients, MS iron and vitamins, 100 mg l⁻¹ m-inositol, 1 mg l⁻¹ riboflavin, 100 mg l⁻¹ proline and 0.2 mg l⁻¹ IBA; after 1 wk in darkness in this medium, the cultures were transferred to fresh medium without auxin and standard light conditions.

Control shoots of oak and apple were treated as above, but without auxin treatment. All media were brought to pH 5.5–5.6 before autoclaving. Cultures

were kept in a growth chamber with a 16/8-h photoperiod (30 µEm⁻².s⁻¹ from cool-white fluorescent lamps) and day and night temperatures of 24 and 20 °C respectively.

Histology

Basal 0.5–0.7 cm pieces of stem were collected daily up to d 10, fixed in 1:1:18 formalin/glacial acetic acid/50% ethanol, dehydrated, and embedded in paraffin wax (Jensen, 1962). Serial 10-µm thick sections cut with a rotatory microtome were stained with safranin-fast green. Starch and other insoluble polysaccharide and total protein were determined by means of the periodic acid Schiff (PAS) reaction and naphthol blue counterstain. Some apple stem segments were fixed in a 2% glutaraldehyde, 2% paraformaldehyde mixture in 0.05 M phosphate buffer (pH 6.8), dehydrated and embedded in LKB Histo-resin as described by Yeung and Law (1987), after which 2-µm thick sections were cut with glass knives on an ultramicrotome and stained by the PAS reaction/ naphthol blue method.

Determination of peroxidase activity

Control and treated oak shoots (100 mg) were lyophilized and homogenized for 2 min in a mortar with 2 ml phosphate buffer (pH 6.1), the homogenate was centrifuged at 10 000 g for 10 min at 4 °C, and the resulting supernatant filtered through a Biogel P2 column to remove phenolics. Peroxidase activity in the filtrate was determined spectrophotometrically at 25 °C by monitoring the formation of tetraguaiacol at 420 nm.

RESULTS

Oak

Morphology

Thickening of the shoot base was first visible 5 d after auxin treatment, and by d 7 the epidermis of some shoots had ruptured to reveal spongy white tissue beneath. Roots began to emerge on d 8, and 4 wk after auxin treatment the percentage of shoots with roots had increased to 85–90%, with an average of 3.4 roots per rooted shoot.

Anatomy

On d 0, before auxin treatment, transverse sections of NL3 shoots exhibited a practically contin-

uous vascular ring together with a number of elements of xylem and secondary phloem. The primary phloem was surrounded by a ring of sclerenchyma formed by groups of 12–18 thick-walled fibres separated by regions of parenchymatic tissue (fig 1a). Cortical, vascular and medullary parenchymal cells had high starch contents.

During the first 1–2 d after auxin treatment, the cortex thickened somewhat and a secondary or phellogenetic cambium began to produce new cell layers in the outermost zone of the shoots. By d 2, a number of cells in the cambial zone or the vascular parenchyma, and the interfascicular spaces (fig 1b), exhibited dedifferentiation characterized by denser cytoplasm and enlarged nuclei with prominent nucleoli; some of these cells had also begun to divide on d 2. Mitosis increased considerably during d 3 and 4 (fig 1c, 1d), so that after d 4 groups of meristematic cells apparently corresponding to Torrey's (1966) meristemoids were observed; these cells were small and isodiametric, and had prominent nuclei staining intensely with naphthol blue (fig 1e). By d 5 or 6, well-defined pointed root primordia had formed, and were generally located at the level of the sclerenchymal ring. During this stage, total protein content increased in the regions with active meristems, while starch and insoluble polysaccharide levels fell. The shoot epidermis was ruptured and cortical parenchymal cells became enlarged and dispersed throughout the regions (fig 1f); the presence of these parenchymal cells was responsible for the spongy macroscopic appearance of the tissue revealed by the ruptured epidermis.

The structure of the stem in control shoots remained practically identical to that observed on d 0. Though in some control shoots, cortical parenchyma also proliferated, and sometimes broke through the epidermis, these parenchymal cells were smaller than in IBA-treated shoots.

By d 7, the primordia developed in auxin-treated shoots had reached the cortex and the first vascular elements (tracheids) had developed. Connection between the vascular systems of root and shoot was generally complete before root emergence.

Peroxidase activity

During the first 4 d in rooting medium, peroxidase activity in NL3 shoots that had received auxin treatment was similar to that in untreated controls (fig 2). Subsequently, peroxidase activity increased in treated shoots, reaching a maximum

of 350 $\mu\text{m}/\text{mg}$ protein on d 6, and then stabilized. In the untreated shoots peroxidase activity declined.

Apple

Morphology

As in oak, the basal region of the shoots became swollen 6–7 d after rooting treatment began, and small quantities of callus were formed on the cut surface. From d 10 to the end of culture (3 wk), the percentage of auxin-treated shoots with roots rose to a final value of 65–75%, with a mean of 4.5 roots per rooted shoot, whereas < 16% of untreated shoots had formed roots.

Anatomy

On d 0, shoot sections showed a ring of collateral vascular bundles. The vascular cambium, with the cambial derivatives which developed gradually to assume the characteristics of the cellular elements of the secondary xylem and phloem, was visible. A discontinuous ring of sclerenchyma surrounded the primary phloem (fig 3a). As in oak, root formation in different shoots was not perfectly synchronous, but a similar sequence of events was apparent. The first changes, the appearance of cambial and vascular parenchyma cells with denser cytoplasm and more pronounced nuclei, were noted 1 or 2 d after beginning auxin treatment, at the same time as the first mitotic divisions (fig 3b). As in oak, some of these dedifferentiating cells were observed in interfascicular locations. From d 3–4 on, the number of dividing cells increased, and groups of meristematic cells (meristemoids) were formed in the phloem region near the cambium (fig 3c). By d 5–6, the meristemoids had begun to become individualized, the plane of cell divisions giving rise to the typical pointed shape of the root primordium. The growing primordia reached the sclerenchymal ring on d 7–8, after which they continued to grow through the cortex. Cortical parenchymal cells enlarged less and were not as dispersed as in oak, so that stem structure was better preserved. Periclinal divisions of cells in the outer layers of the primordium formed a tissue layer that subsequently gave rise to the root cap. As in oak, the connection between the vascular systems of primordium and shoot was usually complete before the

Fig 1. Transverse sections of microcuttings of oak during root formation. **a, b** : Anatomy of the shoot at the time of excision; the inset in fig 1b shows the interfascicular region where some of the first mitotic divisions occur (x 71.5, x 226). **c, d** : d 3, activation of cells and (arrow) first mitotic division in the interfascicular parenchyma (x 452); **e** : d 4, meristemoids (PAS-naphthol blue stain, x 178). **f** : d 6, root primordia and (arrow) disorganized cortex (x 28).

Fig 2. Peroxidase activity during *in vitro* rooting of *Quercus robur* clone NL3.

emergence of the root (fig 3d). The starch content of auxin-treated shoots fell, while total protein content increased significantly after d 4. The structure of untreated control shoots hardly changed during the study period.

DISCUSSION AND CONCLUSION

The anatomical events leading to adventitious root formation have been described as a series of 3 or 4 stages. Hartmann *et al* (1990) divided the process of rooting into dedifferentiation, the formation of root initials, the differentiation of root primordia, and finally the growth and emergence of the root. The sequence of anatomical changes observed in this work during the formation of roots in oak and apple microcuttings was very similar to that reported for the *in vitro* rooting of chestnut (Vieitez *et al*, 1981) and camellia (Sarmartin *et al*, 1986). Root initiation involves the dedifferentiation of specific cells, leading to the formation of root meristems (Hartmann *et al*, 1990).

An interesting question is what determines the boundaries of the organogenic domains. Thorpe (1980) suggested that the organized development leading to *de novo* organogenesis begins with changes in a single cell that becomes activated. The region of the tissue in which cells become activated is thought to depend in part on

physiological gradients of substances entering the shoot from the medium (Ross *et al*, 1973). In a study on *Hedera helix*, Geneve (1991) coined the term "competent root-forming cells" (CRFC) to describe the cells involved in direct initiation of root primordia; such cells are considered to be competent to form adventitious roots in the presence of an exogenously applied or endogenously accumulating inducer (usually auxin) which initiates polar cell divisions eventually leading directly to the formation of a root primordium. Tripepi *et al* (1983) provided evidence concerning early activation events in CRFCs. They showed that throughout the hypocotyl of mung bean cuttings treated with radiolabelled uridine or thymidine there was an increase in both DNA and RNA synthesis in specific cells associated with sites of potential formation of root primordia. Species exhibiting the direct root formation pattern are supposed to possess CRFCs.

In the oak and apple clones studied in our work, the first signs of the formation of adventitious roots were the appearance of larger nuclei and denser cytoplasm in cells located in and adjacent to the vascular bundles near the cambium. Similar findings were reported by Hicks (1987), who observed that in apple the tissues in and around the vascular strands became activated, and that meristemoids formed from certain of these cell populations lying outside the xylem.

Fig 3. Transverse sections of microcuttings of apple during root formation. **a** : anatomy of the shoot at time of excision (x 31). **b** : d3, activation of cells and (arrow) first mitotic divisions in cambial and phloem regions (x 492). **c** : d 4, meristemoid (arrow) (x77). **d** : d10, root emerging from the shoot; note the vascular connexions (arrow) between the main vascular system of the shoot and that of the roots (x 39).

Again, Zhou *et al* (1992) reported that in microcuttings of the easy-to-root *Malus* clone M26, adventitious roots were initiated directly in cambial derivatives (whereas in the difficult-to-root clone

EMLA9, continuous divisions of cambial cells first produced rows of cells; some of those in the outer rows then became meristematic after 4 d IBA treatment).

In this study, we found that changes in peroxidase activity during the *in vitro* rooting of oak shoots exhibited a pattern partly similar to those reported for other woody species. We observed an increase during the rooting treatment but not the characteristic decrease, probably due to measuring peroxidase activity in purified extracts. According to some authors (Moncousin and Gaspar, 1983; Moncousin *et al*, 1988; Gaspar, 1989), the peroxidase activity peak marks the end of the "induction" stage of root formation. In *Sequoiadendron giganteum* the peroxidase peak is reached on d 17, when the meristemoids are formed (Berthon *et al*, 1987); in oak, the maximum peroxidase activity is reached on d 6, when root primordia have already been formed (apparently a couple of days after the formation of meristemoids).

Thus intense mitotic activity, coupled to elaborate metabolic changes, appears to be an essential prerequisite for root formation (Haissig, 1986; Torrey, 1986). Such findings are in keeping with the hypothesis that the initiation of organized development *in vitro* involved a shift in metabolism both before and during the actual development process (Thorpe, 1980). The identification of these changes would lead to a better understanding of the formation of adventitious roots.

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